INTRODUCTION

Lung cancer accounts for more than 1.5 million deaths worldwide annually, with 80% mortality within a year of diagnosis. In addition to developed countries, lung cancer has become prevalent in developing nations like India. India faces about 10% of the world lung cancer incidents. The most common etiological factor for lung cancer is smoking, which is persistently rising in India. Based on size and appearance of malignant cells, lung cancer is categorized into two histological types i.e. non-small cell lung cancer and small cell lung cancer.

In non–small-cell lung cancer (NSCLC), chemotherapy is used as first line treatment which is also reported to improve survival in patients with advanced NSCLC. Usually chemotherapy deals with administration of two drug simultaneously- out of which one is platinum based compound like cisplatin or carboplatin; while other drug has several alternatives including gemcitabine, paclitaxel, Docetaxel, pemetrexed, etoposide or vinorelbine. After patients experienced treatment failure of initial therapy, response to further systemic treatment is approximately10% for single agents. Thus, resistance to systemic therapy does not appear to be an all-or-none phenomenon, but rather a function of molecular characteristics of individual tumors.

Docetaxel, a semisynthetic taxane, was the first agent to show efficacy in the second-line treatment of non-small cell lung cancer (NSCLC), and has since become a mainstay of NSCLC therapy. Docetaxel works primarily by promoting microtubule assembly and polymerization, and through this hyperstabilization, causes cell cycle arrest and death. The primary toxicity of docetaxel is neutropenia, which can be mitigated by weekly administration in selected patients. Less common toxicities are peripheral edema, which can be reduced by appropriate premedication and interstitial pneumonitis.

Docetaxel when administered as such has severe limitation of limited solubility which has to be overcome for effective management of cancer. Although the solubility issue has been mitigated in marketed formulation i.e. Taxotere, it still has restricted use due to presence of Tween 80 which has potential to cause severe side effects. Thus a novel approach, which may overcome the solubility issue and avoid use of harmful agents, is desired which may be achieved by encapsulating the aforesaid drug in proper carrier i.e. nanoparticles. There are reports that encapsulation of drug inside nanoparticles may not completely avoid adverse effects as these can distribute to normal tissue and thus an approach to target desired tissue

was sought.Monoclonal antibodies and tyrosine kinase inhibitors are two types of targeted therapy being used in the treatment of non-small cell lung cancer.

Monoclonal antibody therapy is a cancer treatment that uses antibodies made in the laboratory from a single type of immune system cell. These antibodies can identify receptors on cancer cells or normal substrate having role in growth of cancer cells and attach to them. The result of antibody attachment to such receptors or substrates can be seen in form of cancer cell killing/ inhibition, retardation of cancer cell growth or prevention of metastasis. Monoclonal antibodies are given by infusion and may be used alone or to carry drugs, toxins, or radioactive material directly to cancer cells.

Monoclonal antibodies used to treat non-small cell lung cancer include bevacizumab and cetuximab. Bevacizumab binds to vascular endothelial growth factor (VEGF) and may prevent angiogenesis and thus blocks blood supply and thus nutrition to the cancer cells. Cetuximab binds to epidermal growth factor receptor (EGFR) and works to stop cancer cells from growing and dividing.Tyrosine kinase inhibitors are targeted therapy drugs that block signals needed for tumors to grow. They may be used with other anticancer drugs as adjuvant therapy.Tyrosine kinase inhibitors used to treat non-small cell lung cancer include erlotinib and gefitinib. Crizotinib is one of the agent included in tyrosine kinase inhibitor class that is used to treat non-small cell lung cancer with certain genetic changes.

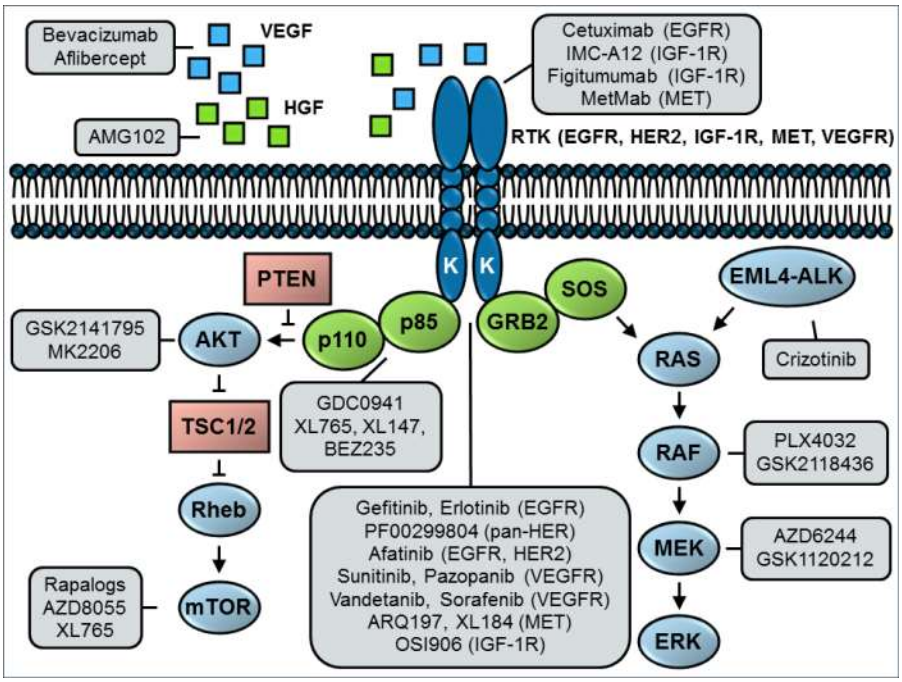


Figure 1: Several targets and their agents for targeted drug therapy

Advantages of targeted drug delivery:

1. Targeted therapeutics binds specifically to the targetantigen.
2. Highly potent agents can be delivered selectively to tumor cells.
3. Wide therapeutic index.
4. Prolonged circulation half-life (conjugate remains stable in circulation)
5. Reduced adverse effects.

Disadvantages:

1. Targeted therapeutics require that the tumor be tested for expression of the antigen.
2. Molecular target may have some normal tissue expression potentially leading to toxicity.
3. Toxic payload may have some premature release.
4. Antibody conjugate may not reach the target cells in sufficient concentration to be lethaldue to physiological, cellular and immunological barriers.
5. Antigen expression could be heterogeneous, especially in solid tumors.

Polymeric Nanoparticles

These nanoparticulate structures encompass both nanospheres and nanocapsules.Nanospheres are amongst the simplest NP formulations, consisting simply of a solidmatrix of polymer, whist the nanocapsule contains an aqueous core. The formulationused is essentially dependent on the solubility of the drug substance; poorly watersolubledrugs are more readily encapsulated within the nonaqueous environment innanospheres, while water-soluble and labile drug substances, such as DNA andproteins/peptides, are more easily encapsulated within nanocapsules.

Various polymers have been examined for use in these formulations, includingpoly(acrylamides), poly(esters), poly(alkylcyanoacrylates), poly(lactic acids),poly(glycolic acids), poly(lactic-coglycolic acid) (PLGA), chitosan (copolymer of Dglucosamineand *N*-acetyl-glucosamine) and alginates (copolymer of guluronic acidand mannuronic acid). PLGA is considered by some to beparticularly attractive owing to its proven biocompatibility.

Targeting Strategies for Nanoparticle Drug Delivery

One of the most exciting areas of NP engineering or formulation is NP targeting. Thetargeting of nanoparticulate formulations focuses on both the development of newdiagnostic tools and improving the efficacies of therapeutic agents. Targetingapproaches can be broadly classified into two areas; passive and active targeting.

Passive Targeting

Passive targeting exploits the normal biodistribution that unaltered NPs will takewithin the body. Upon intravenous delivery, plain NPs are rapidly removed fromcirculation by opsonization and macrophage engulfment or accumulate in the liver.Therefore, this clearance can be exploited to treat hepatic disorders such asleishmaniasis, a parasitic disease, or for the targeting of accumulatedmacrophages in atherosclerosis.

Despite these opportunities to exploit opsonization, the treatment of most diseases,in particular tumors, requires the circumvention of this clearance mechanism. Toavoid opsonization and subsequent phagocytosis, NPs coated with PEG have beenwidely investigated and shown to promote significant improvements in bioavailabilityof the particles. The formation of a PEG shield can be achieved by using PEGduring particle formulation or by adsorption of PEG polymer onto preformedparticles. Studies have suggested that particles up to 400 nm can be passively targeted totumors. This is due to the ability of these particles to leach into the diseased tissuethrough the leaky vasculature network that is commonly associated withtumorigenesis; a phenomenon called the EPR effect. The increased permeabilityof the vasculature in tumors is due to incomplete or disordered endothelial celljunctions that are frequently found in rapidly growing tumors. Consequently, tumorvessels are more permeable to nanoparticulate formulations than the well-definedvasculature found in normal differentiated tissue. Furthermore, tumors tend to havepoor lymphatic drainage, leading to further accumulation of the NP at the diseasedsite. Indeed, the clinical usefulness of Doxil has been attributed, at least in part, to theEPR effect with a significant decrease of doxorubicin side effects.

Active Targeting

Active targeting involves the modification of the NP with a targeting moiety. Thismodification is usually on the corona of the particle, introducing a ligand, whichfacilitates the homing, binding and internalization of the formulation to the targetedcells. Although this approach has been used to target normal cells, most research hasfocused on the specific targeting of cells expressing disease-associated biomarkers, asin the case of cancer. Various moieties have been examined as targeting agents,including vitamins, carbohydrates, peptides (e.g., Arg-Gly-Asp, allatostatin,trans-activating transcriptional activator) and proteins (e.g., lectins andtransferrin). However, the majority of research till date has focused on antibodies.

Antibody-targeted Nanoparticles

Nanoparticle-mediated drug delivery research has examined a full spectrum of nanoparticles in recent research that can be used in diagnostic and therapeutic cancer applications. Antibody–nanoparticle conjugates have the potential to elicit effective targeting and release of therapeutic targets at the diseased site, while minimizing off-target side effects caused by dosing of normal tissues.

While tremendous innovative advances in our understanding of the molecular and cellular basis of cancer, conventional front line cancer treatment remains centered on systemic chemotherapy and radiotherapy. The improved formulation and delivery of chemotherapies has the potential to address these current hurdles in the effective medication of the diseased tissue. The application of nanoparticle (NP) delivery systems has been extensively studied for a range of drug molecules, with various parameters such as size, entrapment efficiencies and release profiles examined in depth. Indeed, nanoparticulate formulations of daunorubicin (DaunoXome®; NeXstar Pharmaceuticals, CO, USA) and doxorubicin (Doxil®/Caelyx®, Janssen, UK and Myocet®, Sopherion Therapeutics, Inc., NJ, USA) are used clinically to treat breast cancer and Kaposi's sarcoma.

Despite these advances in NP formulations on drug bioavailability, targeting of these entities has the potential to further improve both the therapeutic effectiveness of drug compounds at the disease site whilst also reducing off-target effects. This targeting can be achieved through the coating of a broad range of molecules on the surface of the NP to achieve targeting.

Antibodies are considered by many as ideal anticancer therapeutic agents and havebeen an area of intense research since customized monoclonal antibody productionwas reported in the mid-1970s. Indeed, antibody production display and screeninginnovations, such as phage display, mean that antibodies can be derived or engineeredto bind with exceptional specificity to a wide range of target antigens. Importantlyhowever, although antibodies are used very successfully as therapeutic agents in theirown right, they also have the ability to be exploited as targeting agents.

The application of antibodies to deliver conjugated agents to disease sites can beutilized for imaging or diagnostic purposes as in the example of radioisotopes forlymphomas. Alternatively, they can be used for the delivery of active therapeuticssuch as cytokines, prodrug activation enzymes (e.g., β-lactamase andcarboxypeptidase) and chemotherapy toxins.

Clinically, immunoconjugates have been used in cancer treatment. For example,gemtuzumab (Mylotarg®; Wyeth, CT, USA) consists of a CD-33 specific

monoclonalantibody conjugated to a calicheamicin, and was used for the treatmentof acute myeloid leukemia. Furthermore, conjugation of radioisotopes withtargeting antibodies has been developed for both imaging (immunoscintigraphy) andradioimmunotherapy strategies.

Antibody–NP conjugates have potential benefits over current approaches as theyhave the ability to circumvent some of the issues associated with direct conjugates,such as the possible inactivation of the drug entity and the necessary release of thedrug once internalized into endosomal/lysosomal vesicles through pH labile orreducible linkers. Furthermore, whereas only stoichiometric ratios of drug to antibody arepossible with direct conjugates, the potential of much higher drug to antibodyratios are possible with antibody–NP complexes; thereby maximizing theconcentration of drug that can be targeted to the disease site.This current background has led to focused interest in the development ofantibody-coated nanoparticulates; both for lipid-based and non-lipid-based NPs.The majority of nanoparticulate antibody-targeting research has focused onantitumor strategies, using the antibody to target cell-surface markers of disease thatare frequently upregulated or expressed specifically on either the tumor or tumorassociatedcells. In the following sections, the main targets examined for antibody–NPconjugates till date are discussed in more depth.

OBJECTIVES

- Development of stable nanoparticle system of Docetaxel using suitable polymeric carrier
- Active targeting by surface modification with antibody of the anticanceragent Docetaxelto tumour.

RATIONALE

For cancer treatment, use of Docetaxel is limited by its low solubility which has overcome to achieve desired therapeutic effect. For this purpose, drug was incorporated inside nanoparticulate carrier of PLGA. Additionally, biocompatibility of PLGA is well-proven barring problem of toxicity. Furthermore, in order to avoid the off-target effects due to drug distribution to normal tissue, nanoparticles were surface conjugated to cetuximab (EGFR targeting antibody).

HYPOTHESIS

It is hypothesized that the exposure to monoclonal antibodies conjugated nanoparticles would confer a degree of tumor selectivity to approved anticancer drugs which will allow high dose administration with low toxicity potential and thus improve their

therapeutic index. Additionally, active targeting playing side by side would increase the therapeutic efficacy of the targeted nanoparticles.

MATERIAL AND METHODS

Chemicals

Docetaxel was purchased from Sigma Aldrich chemicals, Germany. Poly(D,L-lactide-co-glycolide) (PLGA, copolymer ratio 50:50, Mol weight 34,000 Da Inherent Viscosity [I.V] 0.41) was purchased from Lactel Polymers, Inc. (USA). Polyvinyl alcohol (PVA, average MW 31,000–50,000) was purchased from Sigma Aldrich chemicals, Germany, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Sigma–Aldrich, Germany, N-hydroxysuccinimide (NHS), sodium chloride, disodium hydrogen phosphate and potassium chloride were obtained from Sigma Aldrich chemicals, Germany. Potassium hydrogen phosphate was purchased from Merck Millipore, Mumbai. Chloroform and acetonitrile were purchased from Merck Millipore, Mumbai, India. All other reagents used were of analytical grade from Merck Millipore, Mumbai, India.

Cell line

The human lung carcinoma cell line A549 was purchased from NCCS Pune. A549 cells were cultured in DMEM supplemented with 10% fetal bovine serum (HIMEDIA, India) and 10000 units/ml penicillin, 10 mg/ml streptomycin and 25 µg/ml Amphotericin B (HIMEDIA, India). Cells were maintained at 37°C in a humidified 5% CO2 incubator (IGO150, Jouan, Germany).

Selection of Antibody

Cetuximab is a monoclonal antibody that targets Epidermal Growth Factor Receptor (EGFR). For patients with advanced NSCLC, some doctors may add it to standard chemotherapy as part of first-line treatment. Cetuximab is not FDA approved for use against NSCLC at this time, although it is approved for use against certain other cancers, so doctors can prescribe it for use in NSCLC.

Preparation of PLGA Nanoparticles

PLGA nanoparticles were prepared by a solvent diffusion method. In this method, PLGA and Drug were dissolved in acetone, and the PLGA solution was added to PVP solution through a syringe pump under stirring at 500 rpm in a hood to evaporate acetone in controlled manner.The nanoparticles produced were collected by centrifugation (15,000 rpm, 45 min). The nanoparticles were washed three times using double-distilled water.

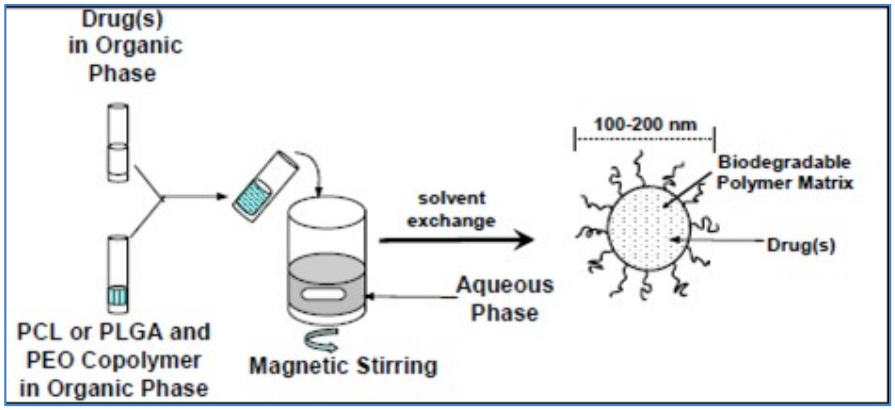


Figure 2: Formation of PLGA Nanoparticles

Formulation of immunonanoparticles

For covalent attachment of cetuximab onto the nanoparticle surface, EDC/NHS chemistry was employed. Concisely, 10 mg of docetaxel nanoparticles was dissolved in 5 ml of 0.02M PBS pH 7.4 followed by addition of 250 ml of 1 mg/ml EDC in 0.02 M PBS, pH 7.4 and 250 ml of 1 mg/ml NHS in 0.02 M PBS, pH 7.4 to the suspension. The samplewas kept at room temperature under agitation for 4 h on a magnetic stirrer plate. Then the sample was ultracentrifuged at 40,000 rpm, 4°C for 20 min to remove unreacted EDC and NHS. The process was repeated three times and the sediment was washed each time with 0.02 M 1 ml PBSpH 7.4. Finally, to dissolve the pellet obtained after centrifugation, 0.02 M 2 ml PBS pH 7.4 was added followed by addition 500 ml of cetuximab (100 mg/ml). The solution was again kept under stirring for 2h on a magnetic stir plate at room temperature and then incubatedovernight at 4°C. After 24 hours ultracentrifugation at 40,000 rpm, 4°C for 20 minutes was carried out to remove unconjugated cetuximab. The process was repeated three times for complete removal of unconjugated cetuximab, the sediment being washed each time with 0.02 M 1 ml PBS pH 7.4. The supernatant was collected and estimatedfor unconjugated cetuximab and the nanoparticleswere lyophilized for further studies.

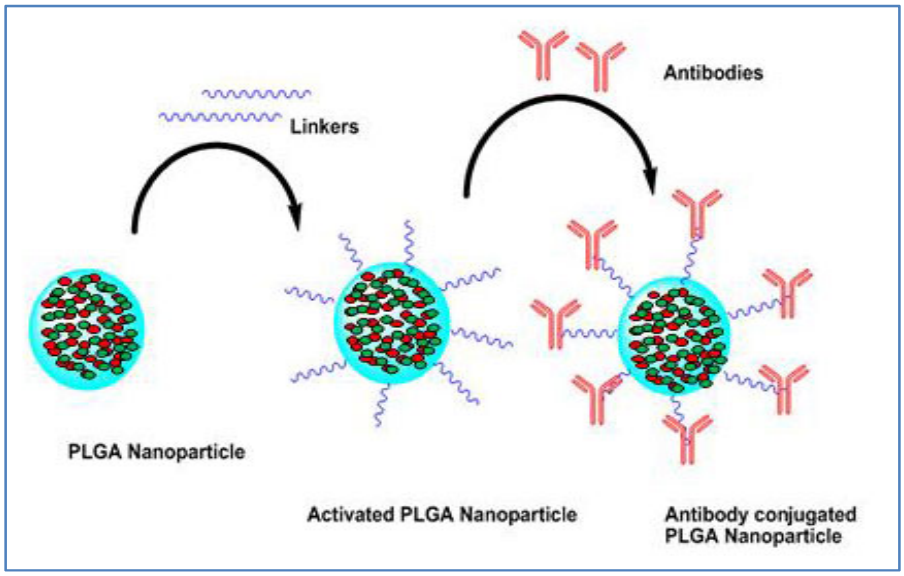


Figure 3: Formation of immuno-nanoparticles

CHARACTERIZATION

Particle size and Zeta potential

Particle size and size distribution of nanoparticleswas determined by dynamic light scattering with a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Sample was diluted in distilled water and sample cuvette then placed in zetasizer. Sample was stabilized for two minutes at 25 °C and reading was measured. The average particle size was measured after performing the experiment in triplicate. The zeta potential of developed nanoparticles was determined using same instrument which calculates the zeta potential by Smoluchowski's equation from the electrophoretic mobility at 25 °C.

Entrapment Efficiency and Drug Loading

Entrapment Efficiencyand drug loading in the NPs was determined after separating the NPs from the aqueous supernatant (containing non-entrapped Docetaxel) by centrifugation at 25000 rpm for 30 min. The supernatant was diluted with appropriate amount of distilled water and analyzed for the amount of unentrapped drug by HPLC after filtration through 0.22 µ and appropriate dilution with mobile phase. Acetonitrile was added to the pellet to dissolve the polymer and centrifuged. The docetaxel peak was measured at a wavelength of 227nm and quantitatively determined by comparing with a standard curve.

Entrapment Efficiencywas estimated by calculating amount of drug entrapped in NPs with respect to total drug added during preparation of formulation. The % EE was calculated according to following formula:

$$EE\ (\%) = (TD\text{-}FD/TD) \times 100$$

Where, TD is total amount of drug added and FD is amount of drug in supernatant Drug loading was calculated as follows,

$$\text{Percentage drug loading} = A/B \times 100$$

Where A is the drug content in the NPs and B is the weight of NPs.

Analytical method

Preparation of standard stock solution

Accurately weighed quantity of Docetaxel working standard (20 mg) was transferred to a 100 ml volumetric flask and 50 ml methanol was added into it. The resultant solution was sonicated to dissolve. The final volume was made upto 100 ml with methanol to obtain a standard stock solution (0.2 mg/ml). An aliquot (3.0 ml) was diluted to 100.0 ml with diluent to obtain a working standard solution of Docetaxel (6 µg/ml).

Preparation of calibration curve

Standard stock solution was diluted to obtain final concentration ranging from 2 to 10 µg/ml.Analytical method for Docetaxel was developed by using different column and different mobile phase. Phenomenex C8 column was found to give better peak shape and lower retention time. Docetaxel was well separated and free from interference of placebo.

Recovery study and intermediate precision study was performed for Docetaxel loaded PEGylated PLGA nanoparticles, and it was found 99.8% with 0.57% RSD at 100% level. Intraday and interday precision assay was found 99.9% and 100.2% with 0.91% RSD and 0.42% respectively.

SEM study

The nanoparticulate dispersion was lyophilized and sample was observed for surface morphology using Scanning electron microscope JSM-5200 (Tokyo, Japan) which was operated at 10 kV.

Differential Scanning Calorimetric (DSC) Studies

All the samples were dried in desiccators for 24 h before thermal analysis. DSC study of pure drug, PLGA, physical mixture of drug: polymer (1:1) and drug loaded NPs was performed in order to characterize the physical state of drug in the NPs. Thermograms were obtained using DSC-60 (Shimadzu, Japan). Dry nitrogen gas was used as the purge gas through the DSC cell at a flow rate of 40 ml/min. Samples (4 - 8 mg) were sealed in standard aluminum pans with lids and heated at a rate of 10 ^{0}C /min from 20 to 300 ^{0}C.

Drug release study

Drug release study was performed using dialysis bag for 24h. 1 ml of the nanoparticulate formulation was filled in dialysis bag and dipped in receptor media comprising phosphate buffer saline pH 7.4 and methanol in ratio of 7:3. 1 ml of sample was withdrawn periodically and fresh media was replaced to maintain sink condition. The sample was analysed using HPLC and % drug released was calculated and plotted against time to get release curve.

In-vitro cytotoxicity (MTT Assay)

The cytotoxicity of different formulations including drug solution, drug loaded nanoparticles and immunonanoparticles was determined using 3-(4,5-dimethylthiazole-2-yl)-2,5- di-phenyl tetrazolium bromide (MTT;HIMEDIA, India) assays. Cells to be assayed were seeded onto 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were treated with respective formulations. After 6h incubation with formulations, transfection media was replaced by fresh DMEM media containing 10% of FBS and antibiotics. The cells were incubated for 48 h and successively 20 µl of a 5 mg/ml MTT solution was added to it. After incubating for 4h, the culture medium was removed and 200 µl of a DMSO (Sigma, USA) was added. The reduction of viable cells count was measured colorimetrically at awavelength of 570 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Rad, USA). Cell viability of each group was expressed as a percentage relative to that of control cells. Graph of log concentration vs. % cell viability was plotted and IC_{50}value was calculated using Graphpad Prism software.

Cell uptake study

Cell uptake was seen by confocal microscopy. Briefly, 1 x 10^4 cells/well were seeded on coverslip placed in 6-well plate. After incubation for 24h, the cells were treated with different formulations loaded with coumarin and allowed to incubate for 6h. The cells were then washed thrice with PBS pH-7.4 and fixed using 4 % paraformaldehyde. DAPI staining was carried out to stain the nuclei of cells and the coverslips were mounted on slide using glycerol. These slides were observed under confocal microscope and cell uptake of different formulations was compared.

Additionally quantitative cell uptake study was performed by flow cytometry. Briefly, cells were seeded in 6-well plate, incubated with different formulations and then trypsinized and harvested cells were analysed by Fluorescence Activated Cell Sorter.

Apoptosis study

For apoptosis study, 1 x 10^5 cells/well were seeded in 6 well plate and after 24h cells were harvested by centrifugation and resuspended in 100 µl annexin binding buffer. To the resulting cellular dispersion annexin V and propidium iodide were added and incubated for 15 min. This was then analysed using flow cytometry.

Cell cycle analysis

1 x 10^5 cells/well were seeded in 6-well plate and after 24h were treated with respective formulations, trypsinized and harvested by centrifugation. The cells were fixed by treating them with 70 % ethanol for 1h at 4 ^{0}C and were lysed using PBS containing 0.2 % triton X-100. Then it was treated with RNAse to remove RNA present in cell lysate as it may also react with propidium iodide. Subsequently it was incubated with propidium iodide and observed using flow cytometry.

Stability Studies

Stability of Docetaxel loaded NPs in terms of drug content and particle size distribution was monitored for 3 months at 2-8 ^{0}C and RT (25-30 ^{0}C). Periodically, samples were withdrawn and the particle size as well as drug content was determined

Statistical Analysis

Statistical analysis of data was performed by ANOVA using Graphpad Prism software (version 6.0). p value<0.05 was considered significant.

RESULTSAND DISCUSSION

One of the greatest challenges of drug delivery involves defining the optimal targeting agent to selectively and successfully transport drug to cancerous cell. There have been numerous investigations aimed at developing more efficient systems for the site specific delivery of drugs. One strategy involves use of tumor-specific antibodies against specific antigens as targeting moieties which can be conjugated onto the nanoparticulate surface for efficient delivery of drug.

EGFR was found to be overexpressed in non-small-cell lung cancer (NSCLC) and associated with increased tumor proliferation, poor differentiation, higher incidence of metastases to lymph nodes and a worse prognosis. The mechanism responsible for EGFR overexpression is largely unknown and gene amplification is only rarely involved in NSCLC. Thus, in order to create agents capable of both drug delivery and molecular targeting, we have developed antibody targeted nanoparticles endowed with covalently attached mAbs against EGFRs to target lung cancer cells. The conventional methods of synthesizing targeted nanoparticles involve formulation and characterization of drug encapsulated nanoparticles

followed by surface modification with the targeting moiety. The fate of a drug after administration in vivo depends mainly on the physicochemical properties of the drug and on its chemical structure, thus physicochemical characterization of drug loaded nanoparticulate system is highly important.

Characterization of NPs and Evaluation of drug contents

Size of the NPs is important for establishing drug delivery strategies to specific sites of the body. Smaller NPs (~100nm) may be prone to minimize the particle uptake by nontargeted cells, including their premature clearance by the MPS (mononuclear phagocytic system). The resulting PLGA-NPs were sized at 108.3±5.9 nm (mean± SD; n=3). Additionally, lower polydispersity index indicated monodisperse formulation.

Zeta potential is an important factor to determine the stability of the NPs in dispersion and also plays an important role in the interaction between the cell membrane and the NPs. PLGA nanoparticles exhibited highest zeta potential (-39.7 mV ± 1.6) due to presence of free carboxyl end group on particle surface.

Entrapment Efficiency and Drug Loading

Drug contents also play a critical role in targeted drug delivery. The PDE of the optimized nanoparticle was found to be 70.28±1.203%. The reason behind such high entrapment efficiency can be attributed to the lipophilic nature of drug. Nanocarriers have also been reported to give high entrapment efficiency for water insoluble drugs.

Topology of nanoparticles as observed by SEM analysis confirmed the smooth and spherical nature of Docetaxel loaded PLGA nanoparticles.

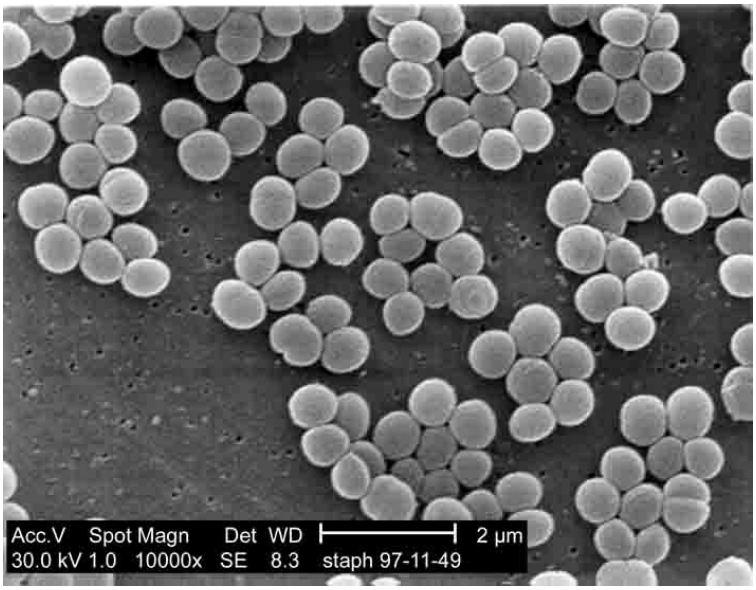


Figure 4. SEM image of nanoparticle

Differential Scanning Calorimetric (DSC) Studies

DSC analysis was performed on native Docetaxel, void nanoparticles and Docetaxel loaded nanoparticles. DSC thermograms help to know the nature of the encapsulated drug in the nanoparticlesas the physical state of the drug in the polymeric matrix could influence its release characteristics. It is observed that the endothermic peak of PLGA is at 54 ^{0}C. as PLGA exhibited a glass transition temperature (Tg) at 54^{0}C. The endothermic peak of native Docetaxel was found approximately at 180 ^{0}C. This characteristic peak was not observed in drug loaded nanoparticles. The absence of detectable crystalline domains of Docetaxel in drug loaded nanoparticles clearly indicates that Docetaxel encapsulated in nanoparticles was in the amorphous or disordered-crystalline phase or in the solid-state solubilized form in the polymeric matrix. This disorderedcrystalline phase of Docetaxel inside the polymeric matrix would help in sustained release of the drug from the nanoparticles. Presence of drug in crystalline form inside nanoparticles hampers its release as such large sized molecules cannot diffuse from the small pores of the nanoparticles. However, if the drug is in amorphous or in disordered-crystalline phase easy diffusion of drug molecules can occur through the polymeric matrix leading to a sustained release of the encapsulated drug. It was also observed that the peak of PLGA was slightly shifted to 58°C in drug loaded nanoparticles as compared to that of void nanoparticles due to presence of Docetaxel inside nanoparticles.

DSC thermogram of Docetaxel, PLGA, physical mixture (of Docetaxel, PLGA) and lyophilized formulation are presented in figure 5, figure 6 and figure 7 respectively.

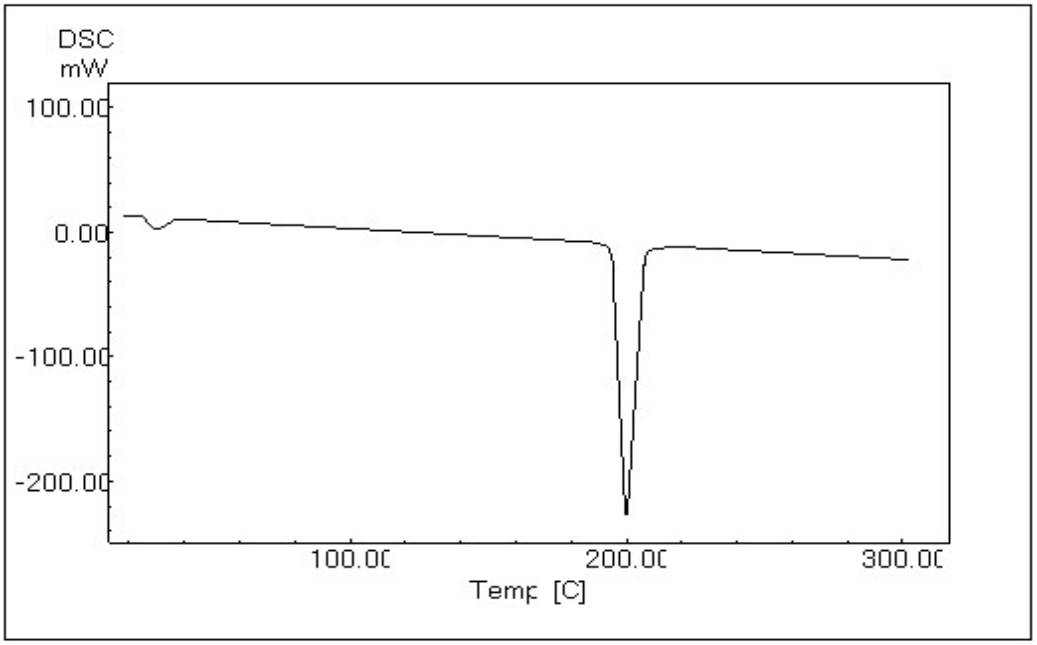


Figure 5 DSC thermogram of Docetaxel

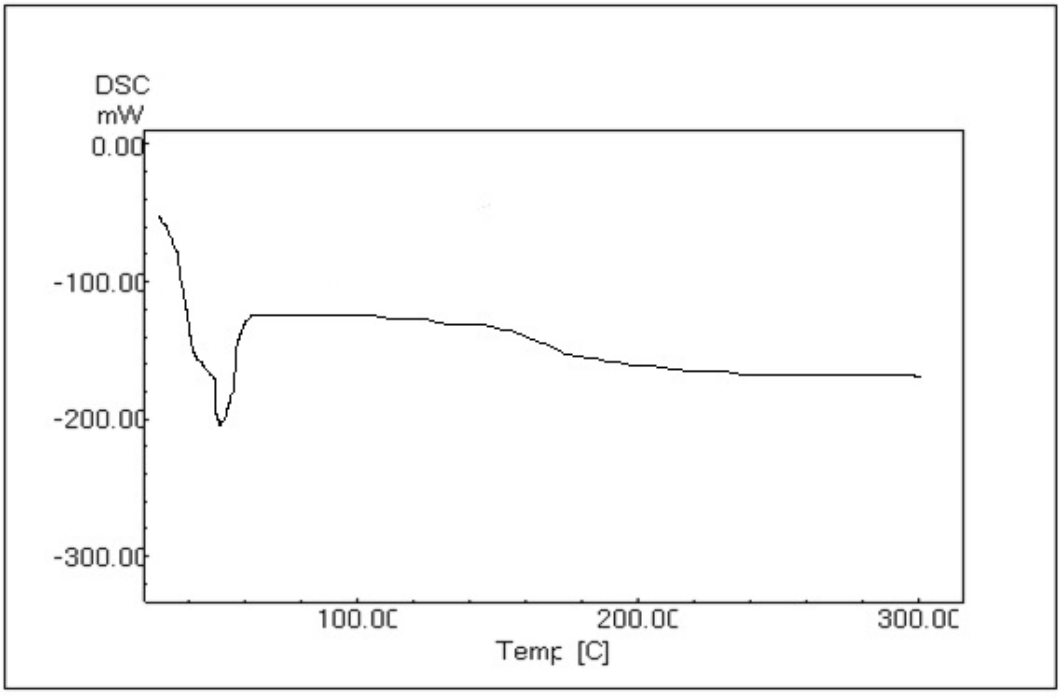


Figure 6 DSC thermogram of PLGA

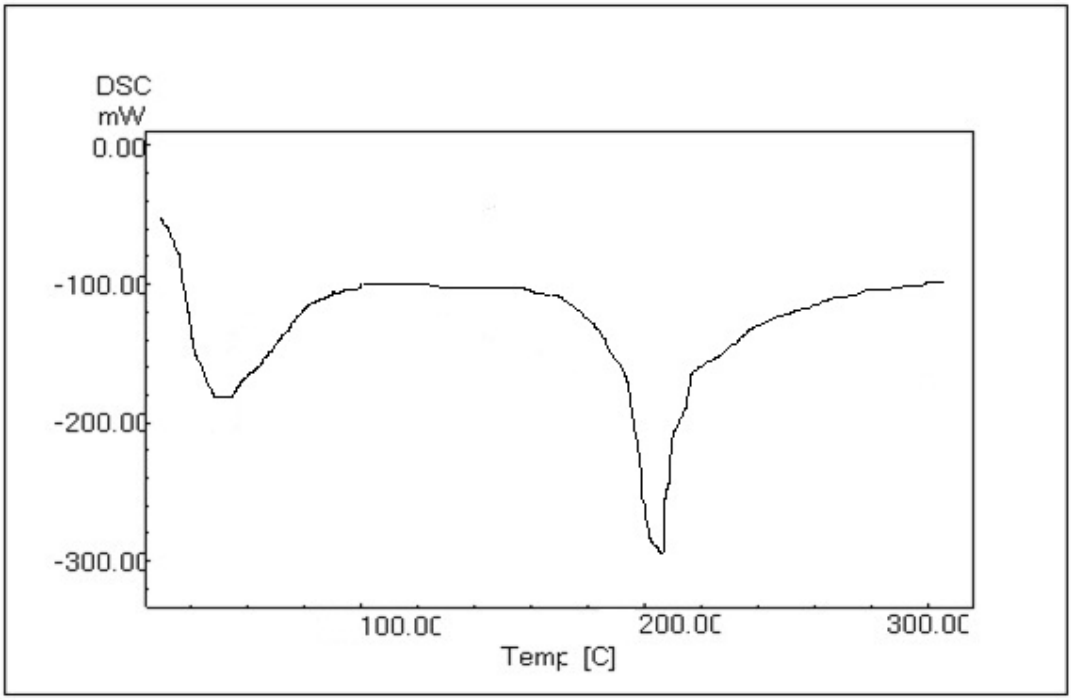
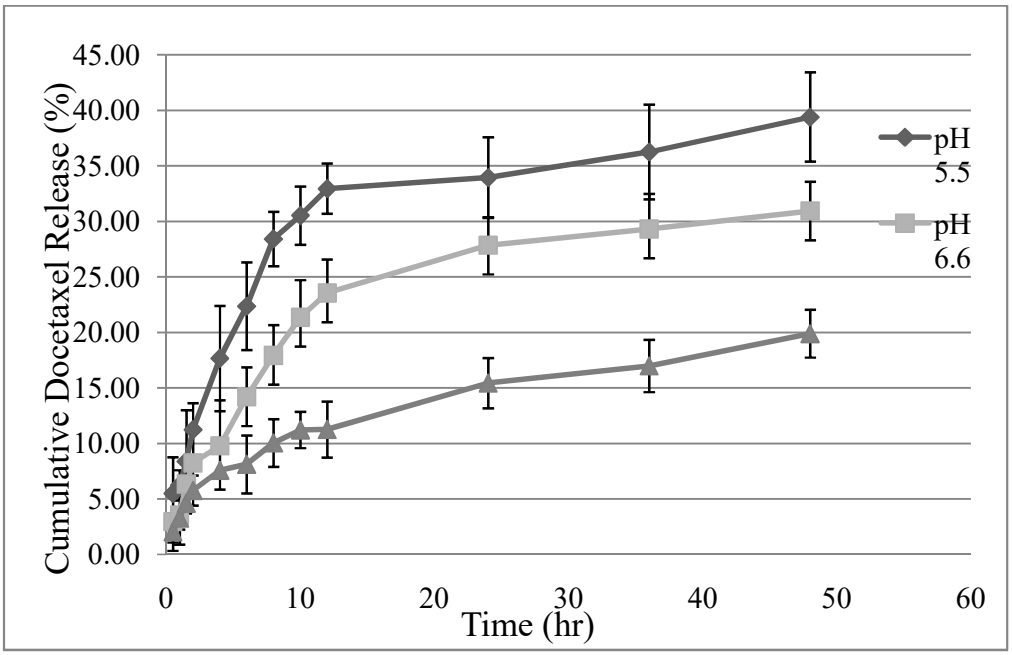


Figure 7 DSC thermogram of Docetaxel and PLGA physical mixture

Drug Release study

Efficient release of encapsulated drug from nanoparticles is an important parameter for developing successful formulations. Docetaxel showed sustained release kinetics from PLGA nanoparticles as shown in figure below. Three conditions of pH namely pH 5.5, 6.6 and 7.4 was used for drug release study to imitate conditions of tumor cells, tumor interstitium and blood or normal cells. Highest release was obtained in pH 5.5 media assuring maximum release of docetaxel in cancer cells. The release decreased in the order, pH 5.5>pH 6.6>ph 7.4. This ensured least release of docetaxel in blood and normal tissues. In pH 5.5 and 6.6 media, initial burst release was observed followed by sustained release characteristic. The initial burst release may be due to the diffusion of docetaxel present at or just beneath the surface of the nanoparticles. The initial burst release was followed by a slower sustain release of docetaxel present inside the core of nanoparticles.



In-vitro cytotoxicity (MTT Assay)

IC_{50} values of different formulations obtained by MTT assay is represented in following table below.

	Drug solution	**Drug loaded nanoparticles**	**Immuno-nanoparticles**
IC_{50} value	3.88 ± 0.34 µM	2.93 ± 0.33 µM	2.20 ± 0.07 µM

MTT results confirmed that targeted nanoparticles exhibited a lower IC_{50} value in comparison to nanoparticles and drug solution. In this study, drug solution demonstrated only transient inhibition of proliferation as compared to Docetaxel loaded nanoparticles and immune-NPs. This could be explained on the basis of the differences in the cellular uptake. Intracellular uptake of native docetaxel was lower than drug loaded nanoparticles while uptake of conjugated nanoparticles was highest amongst all formulations. Native drug molecules cross cell membrane by diffusion which reaches saturation at certain point and thus limits drug penetration.Conversely, cellular internalization of unconjugated PLGA nanoparticle is mediated by nonspecific endocytosis. It is reported that antiproliferative effect is directly proportional to cytoplasmic drug concentration. It was further demonstrated that nanoparticles rapidly escape the endolysosomes and enter the cytoplasm under the in vitro cell culture conditions. The fraction of nanoparticles that escape the endosomal compartment

seem to remain in the cytoplasmic compartment and release the encapsulated therapeutic agent in a sustained manner as the polymer degrades slowly. Hence, we can anticipate that drug loaded nanoparticles have enhanced therapeutic efficiency than native drug. However, in this study we propose that conjugation of a targeting moiety to nanoparticles further enhances its therapeutic effectiveness to a higher level in comparison to unconjugated nanoparticles and native drug. The higher antiproliferative activity of the immune-nanoparticles observed in our study was achieved because of receptor mediated endocytosis by binding to EGFRs in the cell membrane which aids in the intracellular accumulation of the nanoparticles.

Cell uptake study

The results of cell uptake study confirmed that there was comparatively higher cellular uptake of immunonanoparticles as compared to non-conjugated nanoparticles and free drug. Higher cellular uptake of EGFR conjugated immunoparticles was proportional to EGFR amounts on cell surface and receptor mediated endocytosis pathway. Besides the above mechanism, other reasons may also existwhich can explain higher uptake values of immunoparticles. Sahoo and Labhasetwar have shown that more amounts of the internalized unconjugated nanoparticles underwent exocytosis during the first 2 h as compared to Tf-conjugated nanoparticles. Hence, greater intracellular uptake of EGFR conjugated immunoparticles may also be due to lower exocytosis and greater intracellular delivery of drug. Enhanced intracellular uptake of immuno-NPs was further confirmed by confocal microscopy where enhanced fluorescence of the immunoparticles showed that they were internalized more in comparison to native dye and unconjugated 6-coumarin nanoparticles.

Apoptosis study

The historical development of the cell death concept is reviewed with special attention given to the origin of terms like necrosis and apoptosis. The explosion of studies on apoptosis in recent years has described it as a regulated, controlled and complex process of autonomous cellular dismantling, also referred as programmed cell death. Cells undergoing apoptosis show characteristic morphological and biochemical features. These features include chromatin aggregation, nuclear and cytoplasmic condensation. On the opposite, necrosis represents a passive consequence of gross injury to the cell. It is morphologically different from apoptosis, and its physiological consequences are also very different from those of apoptosis.

Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. To determine if inhibition of EGFR

resulted in apoptosis of A549 cell line, apoptosis studies was conducted using Annexin V staining procedure. Immuno-NPs were able to cause more apoptosis and necrosis in A549 cell lines ascompared to drug loaded nanoparticles and native drug following site specific sustained delivery pattern. Additionally it was observed that dose elevation of drug results in reduction of the degree of apoptosis as tumor necrosis increases. In our study, native docetaxel diffuses and accumulates directly at site of action at a higher concentration thus resulting in necrosis. However, nanoparticles show a slow sustained release phenomenon and at a lower dose apoptosis signals may be activated leading to a higher number of cell deaths. On contrary targeted therapy showed mixed results, with cells being present both in apoptosis phase as well as in necrosis stage. Targeted delivery and better uptake of immunoparticles results in greater accumulation of drug loaded nanoparticles inside tumor tissue. Rapid release of surface adsorbed drug (burst release) from nanoparticles is responsible for causing necrosis initially in the A549 cells. However, in later stages slow and sustained release of drug molecules is responsible for eliciting apoptotic signals causing death by apoptosis.

Sustained cytoplasmic delivery of the drug from nanoparticles coupled with the site specific delivery of docetaxel due to EGFRs have resulted in more enhanced therapeutic potency of the immunoparticles by apoptosis than unconjugated nanoparticles thus supporting the idea that surface modified nanoparticle can serve as effective delivery vehicles.

Cell cycle analysis

Cell cycle analysis demonstrated that the cells treated with docetaxel had a higher proportion of cells in the G1 phase. It was observed that a higher proportion of cells were in the G1 arrest phase following treatment by immuno-NPs and drug loaded nanoparticles than the cells treated with the native docetaxel drug. There are previous reports confirming higher arrest of higher number of cells in G0/G1 phase when treated with nanoparticles as compared to free drug treated cells. However, immuno-NPs inhibiting more number of cells in G1 phase can be explained on the basis of the intracellular drug levels. It can be said that in case of nanoparticle mediated targeted therapy more drug is available at the site of action (following sustained drug release) for a longer period of time than native drug in solution, resulting in greater efficiency of the immunoparticles in arresting cell growth. Immuno-NPs could have facilitated the intracellular delivery of Docetaxel thus resulting in higher inhibition of cell proliferation.

Stability Studies

The stability study of PLGA nanoparticles and antibody congugated PLGA nanoparticles was carried out at 2-8°C / ambient humidity and 30±2 °C/60±5 % humidity for

the period of 12 months. There is no significant increase in particle size at 2-8°C / ambient humidity for pegylated gelatin nanoparticles and PEGylated PLGA nanoparticles. At 30±2°C/60±5 % humidity condition, particle size was increased. Drug retained in nanoparticles (considering initial amount of loaded drug 100%) was also evaluated. Nanoparticles retained more than 95% of drug at both conditions after storage of 12 months.

Particle sizeof and drug retainedin Nanoparticles for period of 12 months at 2-8 °C

Batch	**2-8 °C / ambient humidity**				
	0 Day	1 month	3 months	6 months	12 months
Particle size (nm)	111.7±5.2	114.2±4.4	112.7±4.2	116.5±5.7	115.4±3.8
Drug retained	100%	100.2±0.96%	98.79±0.78%	98.49±1.02%	99.12±1.52%

Particle sizeof and drug retained in Nanoparticles for period of 12 months at 30±2 °C/60±5 %

Batch	**30±2 °C/60±5 % Humidity**				
	0 Day	1 month	3 months	6 months	12 months
Particle size (nm)	111.7±5.2	118.6±16.3	124.9±10.9	131.5±14.9	137.2±13.5
Drug retained	100%	99.68±0.96%	97.02±1.25%	96.56±1.59%	96.01±2.63%

CONCLUSION

Present investigation shows a promising way to treat lung cancer using targeted delivery of docetaxel encapsulated nanoparticles conjugated to cetuximab. The proposed formulation showed enhanced efficacy with reduced side effects and other desirable physicochemical properties. Additionally, the adverse reactions suffered from marketed formulation may be avoided by application of such novel targeted formulation. Thus this formulation can serve as a better alternative for lung cancer treatment after further studies.

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